Increase in ceramide level after application of various sizes of sphingomyelin liposomes to cultured human skin model

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Abstract

Sphingomyelin based liposomes that were sized (or not) by extrusion through a filter with pores of 100, 200, or 400 nm were applied to a three-dimensional cultured human skin model in order to evaluate which size of sphingomyelin liposome was most effective at increasing its ceramide level. The diameters of the sphingomyelin liposomes in PBS were 102.7, 181.0, 224.0, and 380.1 nm. The diameters of the liposomes in the culture medium were 117.5, 199.2, 242.1, and 749.8 nm. The diameter of the small liposomes (<200 nm in diameter) did not change much, at least for seven days. Sphingomyelin liposomes in saline or culture medium were applied to the basal layer side or stratum corneum side of the cultured skin model, and ceramide II, III, V, and VI were then extracted from it. The extracted ceramide molecules were separated by HPTLC, and the concentration of each type of ceramide was quantified using a densitometer. When the small sphingomyelin liposomes (110 or 190 nm in diameter) were applied to the basal layer side, the levels of ceramide III and V were increased. When they were applied to the stratum corneum side, the levels of ceramide II, III, V, and VI were significantly increased compared to those of the PBS applied group, especially after the application of the small sphingomyelin liposomes (110 nm in diameter). Thus, the application of small sphingomyelin liposomes was useful for increasing the ceramide II, III, V, and VI levels of a cultured human skin model.

Keywords: sphingomyelin liposomes, ceramide, skin, three-dimensional cultured human skin model, small liposomes

Introduction

The stratum corneum (SC) is the primary barrier against the permeation of therapeutic drugs and cosmetic ingredients through the skin [1, 2]. The SC has a special structure with keratinocytes acting as bricks and intercellular lipids acting as a mortar-like structure [3, 4]. The intercellular lipids of the stratum corneum include ceramides, cholesterol, cholesterol esters, and fatty acids [5, 6]. Disruption of or a lack of SC lipids decreases the effectiveness of the barrier function of the skin and skin moisturization, causing dry skin and sometimes skin disease. In particular, ceramides, the primary constituents of intercellular lipids, play the most important roles in the barrier function of the SC [7, 8]. It was also reported that the sphingolipid concentration was significantly decreased and the barrier function of the skin was inhibited in atopic dermatitis-patients [8- 10].

Ceramide makes up 30- 40 % of the stratum corneum by weight [11, 12]. The nine major stratum corneum ceramide species are generated through two biosynthesis pathways, glucosylceramide hydrolysis through glcocerebrosidase and sphingomyelin (SPM) hydrolysis through sphingomyelinase. Generally, ceramide II and V are generated from SPM, and ceramide III, IV, and VI are generated from glucosylceramide [9, 13, 14]. SPM is a sphingolipid that is found in the cell membranes of mammalian cells. SPM contains phosphorylcholine and ceramide. It is hydrolyzed by sphingomyelinase in the granular layer of the skin to generate ceramide and phosphocholine [15]. SPM is difficult to formulate in topical formulations and cosmetics, since it is not soluble or stable in aqueous formulations, which are frequently used for cosmetics.

Recently, reconstructed cultured human skin models (cultured skin models) have been researched and developed as a promising alternative membrane for human and animal skins. Three dimensional human skin model such as EPISKIN® is used as various study

materials [16]. In this manuscript, LabCyte Epi-Model which consisted of the human keratinocytes was used as a three dimensional model

We reported that the application of sphingomyelin-based liposomes (SPM-L) to a three- dimensional cultured human skin model increased its type II ceramide content [17]. In the present study, we prepared various sizes of SPM-L and applied them to the aforementioned three-dimensional cultured human skin model to evaluate which size of SPM-L is most effective at increasing its ceramide level.

Materials and Methods

Materials

Sphingomyelin (SPM, form milk) was purchased from NOF Corporation (Tokyo, Japan). Hydroxy and non-hydroxy ceramide standards were obtained from Matreya, LLC (Pleasant Gap, PA, USA). Type III and VI ceramide were obtained from Evonik Goldschmidt GmbH (Goldschmidtstrasse, Essen, Germany). LabCyte Epi-Model (a three-dimensional cultured human skin model) medium and enhanced keratinization medium were purchased from Japan Tissue Engineering Co., Ltd. (Gamagori, Aichi, Japan). Phosphate-buffered saline (PBS) powder was obtained from Sigma (St. Louis, MO, USA). All other reagents were obtained commercially and used without further purification. Silica gel 60 (Merck, Darmstadt, Germany) was used for the HPTLC plate.

Preparation of sphingomyelin based liposomes

SPM-L were prepared using a thin layer film and the freeze-thawing method [18]. SPM (77.95 mg) was dissolved in 1 mL of chloroform (100 mM). The chloroform was then evaporated and dried using a rotary evaporator under 40 °C and 50 hPa, before being stored

in vacuo for at least 1 h. The resulting thin lipid film was hydrated with PBS (pH 7.4). Then, the liposomes were subjected to freezing and thawing for five cycles with liquid nitrogen and extruded five times through polycarbonate double-membrane filters using an extruder (pore-size: 100, 200, or 400 nm; Nucleopore, Costar, Cambridge, MA, USA). Finally, the SPM-L suspension was separated by ultracentrifugation at 250,000×g for 15 min (Himac CS100 GXL, Hitachi, Tokyo, Japan). The resultant liposomal pellets were resuspended in PBS (pH 7.4) or culture medium.

Stability of the diameter of the sphingomyelin based liposomes

The diameters of the SPM-L were measured by dynamic light scattering using a Zetasizer (3000HSA, Malvern, London, UK). The diameters of the liposomes in the PBS were measured at 0, 1, 2, 4, and 7 days, and those of the liposomes in the culture medium were measured at 0, 6, 24, and 48 hours.

Application of sphingomyelin-based liposomes to the basal layer side of the LabCyte Epi-Model and the extraction of various types of ceramide.

One percent of SPM-L of various sizes in physiological saline or saline alone (control) (1.0 mL each) was applied to the basal layer side of the LabCyte Epi-Model for 6 hours. The cell cultures were grown 5 % CO_2 and 37°C. 1.0 mL of the culture medium containing ascorbic acid (final concentration: 25 μg/mL) was then exchanged for enhanced keratinization medium. After 24 hours, the ceramide molecules were extracted from the cultured skin model using 6.0 mL of chloroform: methanol (2:1 v/v).

Application of sphingomyelin-based liposomes to the stratum corneum side of the LabCyte

Epi-Model and the extraction of various types of ceramide.

One percent of SPM-L of various sizes in the culture medium or medium alone (control) (0.3 mL each) was applied to the stratum corneum side of the LabCyte Epi-Model for 7 days. The cultures of LabCyte Epi-model were grown 5 % CO_2 and 37°C. Medium (1.0 mL) was changed every day. After 7 days, 1.0 mL of the culture medium containing ascorbic acid was then exchanged for enhanced keratinization medium in 24 hours.

The extraction method and ceramide analysis

Various ceramides in LabCyte Epi-Model was extracted by using the Bligh and Dyer method [19]. Briefly, Labcyte Epimodel was dissolved in 6 mL of chloroform/methanol (2/1 v/v) and sonicated (70 W, 10 min) by probe type sonicator (Sonifire B-12, Branson Ultrasonics, CT, USA). The ceramide extracted solution was dried by the nitrogen gas, and resolved 0.4 mL of chloroform/methanol (2/1 v/v). Various ceramide extract was prepared.

Ceramide analysis by HPTLC (high performance thin-layer chromatography)

Various ceramide extracts were separated using an HPTLC plate (Silica Gel 60, Merck, Darmstadt, Germany). The HPTLC was developed twice with chloroform: methanol: acetic acid = 190: 9: 1 (v/v). The ceramide molecules were visualized by treatment with 10 % $CuSO_4$, 8 % H_3PO_4 aqueous solution, and heating to 180 °C for 10 min. The amounts of the various types of ceramide on the HPTLC plate were quantitatively determined using a densitometer.

Data analysis

All experiments were performed three to five times, and the data are shown as the

mean ± standard deviation. Dunnett's multiple comparison test was performed for statistical analyses.

Results

Change in the diameter of the sphingomyelin liposomes

The diameters of the SPM-L were analyzed by dynamic laser light scattering. Figure 1a and b show the change in the particle diameter of the SPM-L in PBS and culture medium, respectively. The mixtures (SPM-L and PBS or culture medium) were incubated for different periods (SPM-L in PBS; t= 0, 1, 2, 4, or 7 days, SPM-L in culture medium; t= 0, 6, 24, or 48 hrs) before their diameter change was measured. The diameter of the small SPM-L (110- and 190-nm) did not change in PBS or culture medium. On the other hand, the diameter of the large SPM-L (240-nm and without sizing) was greatly increased in a time dependent manner.

Figure 1

Change in the ceramide contents of the LabCyte Epi-Model after the application of various sized sphingomyelin liposomes to the basal layer side of the cultured skin model.

One percent of various sized SPM-L was applied to the basal layer side of the LabCyte Epi-Model for 6 hours. The skin model was then cultured in enhanced keratinization medium containing ascorbic acid for 24 hours, before the ceramide molecules were separated from the cultured skin model and determined by HPTLC. Figures 2a (ceramide II), 2b (ceramide III), 2c (ceramide V), and 2d (ceramide VI) show the amounts of each ceramide in the cultured skin model. The amounts of ceramide II, V, and VI were all

increased a little by SPM-L application. However, the ceramide level was not influenced by the particle diameter of the SPM-L.

Figure 2

Change in the ceramide contents of the LabCyte Epi-Model after the application of various sized sphingomyelin liposomes to the stratum corneum side of the cultured skin model.

Next, 1.0 % of various sized SPM-L was applied to the stratum corneum side of the epidermis of the LabCyte Epi-Model for 7 days. The skin model was cultured using the same method as described above. The ceramide molecules were separated from the cultured skin model and determined by HPTLC. Figure 3 shows the developed HPTLC plate image. Figures 4a (ceramide II), 4b (ceramide III), 4c (ceramide V), and 4d (ceramide VI) show the amounts of each ceramide in the cultured skin. The amounts of ceramide II, V, and VI were significantly increased after the application of the small SPM-L.

Figure 3

Figure 4

Discussion

In this paper, various SPM-L were applied, and increases in the levels of several types of ceramide were observed in a three-dimensional cultured human skin model. We have reported that a cultured human skin model demonstrated sphingomyelinase activity and that SPM-L application to the three-dimensional cultured human skin model increased the amount of type II ceramide in the cultured skin [17].

At first, the change in the diameter of the SPM-L in PBS or culture medium was examined. The prepared SPM-L were ultracentrifuged and resuspended in PBS or culture medium. The diameter of the small SPM-L (110- and 190-nm) in PBS did not change, at least for seven days. On the other hand, the diameter of the large SPM-L (240-nm and without sizing) increased with time. The surface charge of the present SPM-L was -3.9 mV (data not shown), and it was assumed that the large liposomes became aggregated with the passage of time.

SPM-L were applied to the basal layer of the model in order to evaluate their influence on the barrier function of the stratum corneum. When we applied the small SPM-L (110 or 190 nm in diameter), the levels of ceramide III and V in the model were increased a little. However, the diameter of the liposomes had little influence on the ceramide content of the model. The amount of ceramide generated did not increase much as the liposome diameter increased. It is suggested from the above findings that the interactions of SPM-L with cells are influenced by their diameter. When the SPM-L were applied to the stratum corneum side, the levels of ceramide II, III, V, and VI were significantly increased compared to those of the PBS application group, especially when the small SPM-L (110- or 190- nm in diameter) were applied. It was also suggested that the penetration of the skin by the small liposomes (110–, 190- nm) was greater than that by the large liposomes (240- nm, MLV). In addition, the small SPM-L generated the most ceramide. This effect may have been higher with even smaller liposomes (< 100nm). However, the preparation of small stable liposomes is difficult; therefore, it was not examined in this study.

In the present study, various sized liposomes were applied to the basal layer side or stratum corneum side of a cultured human skin model. As a result, the amounts of ceramide II, III, V, and VI in the skin were increased. Generally, major ceramides are generated

through two pathways (Fig. 4). All species of ceramides can be produced through glucocerebrosidase activity, while only two species (type II and V ceramide) can be generated through the hydrolysis of sphingomyelin by sphingomyelinase [9, 13, 14]. These ceramides are then metabolized to sphingosine and free fatty acids by ceramidase [20]. Thus, the ceramide content of the SC is controlled by three enzymes: glucocerebrosidase, sphingomyelinase, and ceramidase. In this study, ceramide was not only generated by enzymatic reactions after the application of SPM-L. The factors of metabolism systems and/or signal transduction systems may be changed by the application of SPM-L. As a result, not only the levels of ceramide II and V but also those of III and VI were increased by the application of SPM-L. We will investigate the mechanism of ceramide generation in the skin by SPM-L in a future study.

Figure 4

Thus, the present SPM-L are effective at enriching the ceramide level of human skin. In addition, liposome formulations are very useful for the application of sphingomyelin to skin since the lipid itself is difficult to formulate in conventional formulations. Type II and V ceramide are reported to be produced from sphingomyelin in the epidermis in vivo by sphingomyelinase. In a future study, we will investigate the changes in the amounts of other ceramides.

References

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Figure captions

Figure 1 Changes in the diameters of various sized sphingomyelin-based liposomes. Sphingomyelin liposomes composed of 100 % sphingomyelin were incubated in phosphate buffered saline (a) or culture medium (b) (including 10 % FBS) at 37 °C in 5 % CO_2. Symbols: 110-nm (●), 190-nm (■), 240-nm (▲), MLV (○). Each data point represents the mean and standard deviation of at least three independent experiments.

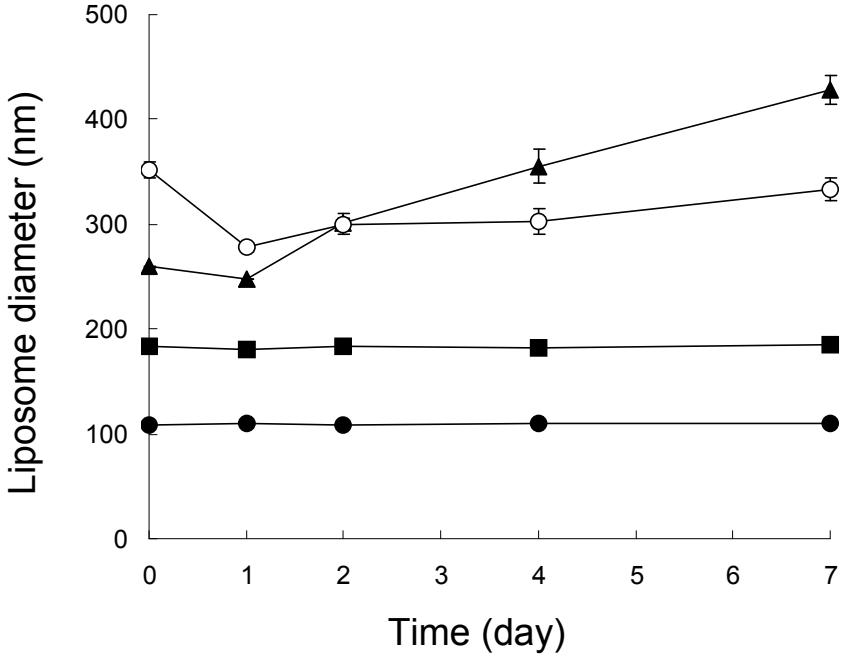
Figure 2 Changes in the ceramide content of the LabCyte Epi-Model after the application of sphingomyelin-based liposomes to the basal layer side of the cultured skin model. (a) ceramide II, (b) ceramide III, (c) ceramide V, and (d) ceramide VI. Sphingomyelin liposomes were applied to the basal layer side of the epidermis for 6 hours after it had been cultured in enhanced keratinization medium containing ascorbic acid for 24 hours. Each data point represents the mean and standard deviation of at least three independent experiments. *, **, and ***: $p < 0.05$, $p < 0.01$, and $p< 0.001$, respectively, compared to the control group. MLV: multilamellar vesicle (without sizing).

Figure 3 HPTLC ceramide image of a LabCyte Epi-model after the application of sphingomyelin-based liposomes to the stratum corneum side. Lane A: control (PBS), Lanes B, C, D and E: MLV, 240-, 190- and 110-nm of sphingomyelin-based liposomes, respectively. MLV: multilamellar vesicle (without sizing).

Figure 4 Changes in the ceramide content of the LabCyte Epi-Model after the application of sphingomyelin-based liposomes to the stratum corneum side of the cultured skin model. (a) ceramide II, (b) ceramide III, (c) ceramide V, and (d) ceramide VI. Sphingomyelin liposomes were applied to the stratum corneum side of the epidermis for 7 days after it had been cultured in enhanced keratinization medium containing ascorbic acid for 24 hours. Each data point represents the mean and standard deviation of at least three independent experiments. *, **, and ***: $p < 0.05$, $p < 0.01$, and $p< 0.001$, respectively, compared to the control group. MLV: multilamellar vesicle (without sizing).

Figure 5 Metabolic pathway of ceramides in the epidermis

(a)


Liposome diameter (nm)
500
400
300
200
100
0
0
1
2
3
4
5
6
7
Time (day)

(b)

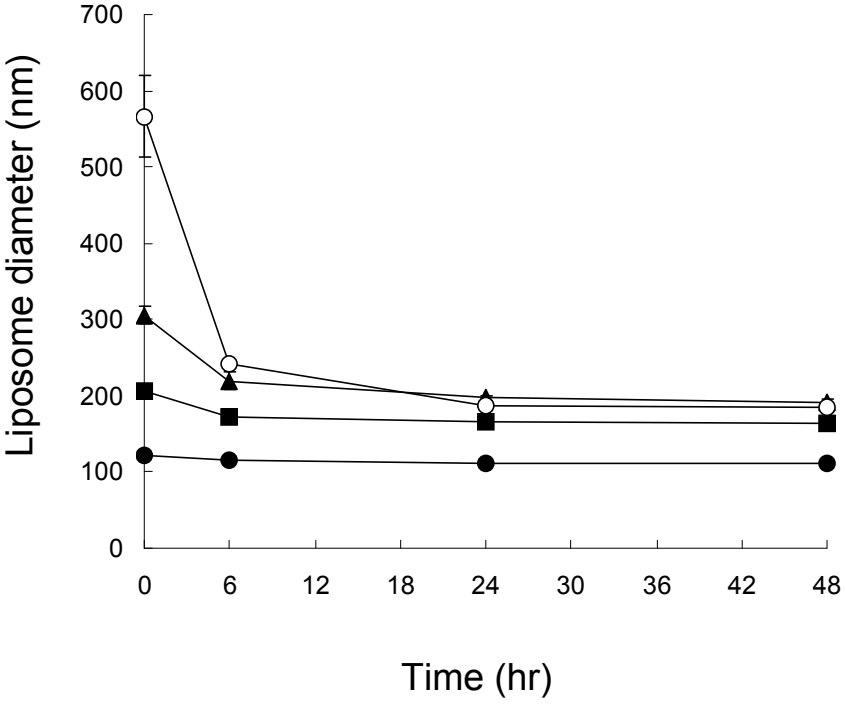

Liposome diameter (nm)
700
600
500
400
300
200
100
0
0
6
12
18
24
30
36
42
48
Time (hr)

Fig. 1

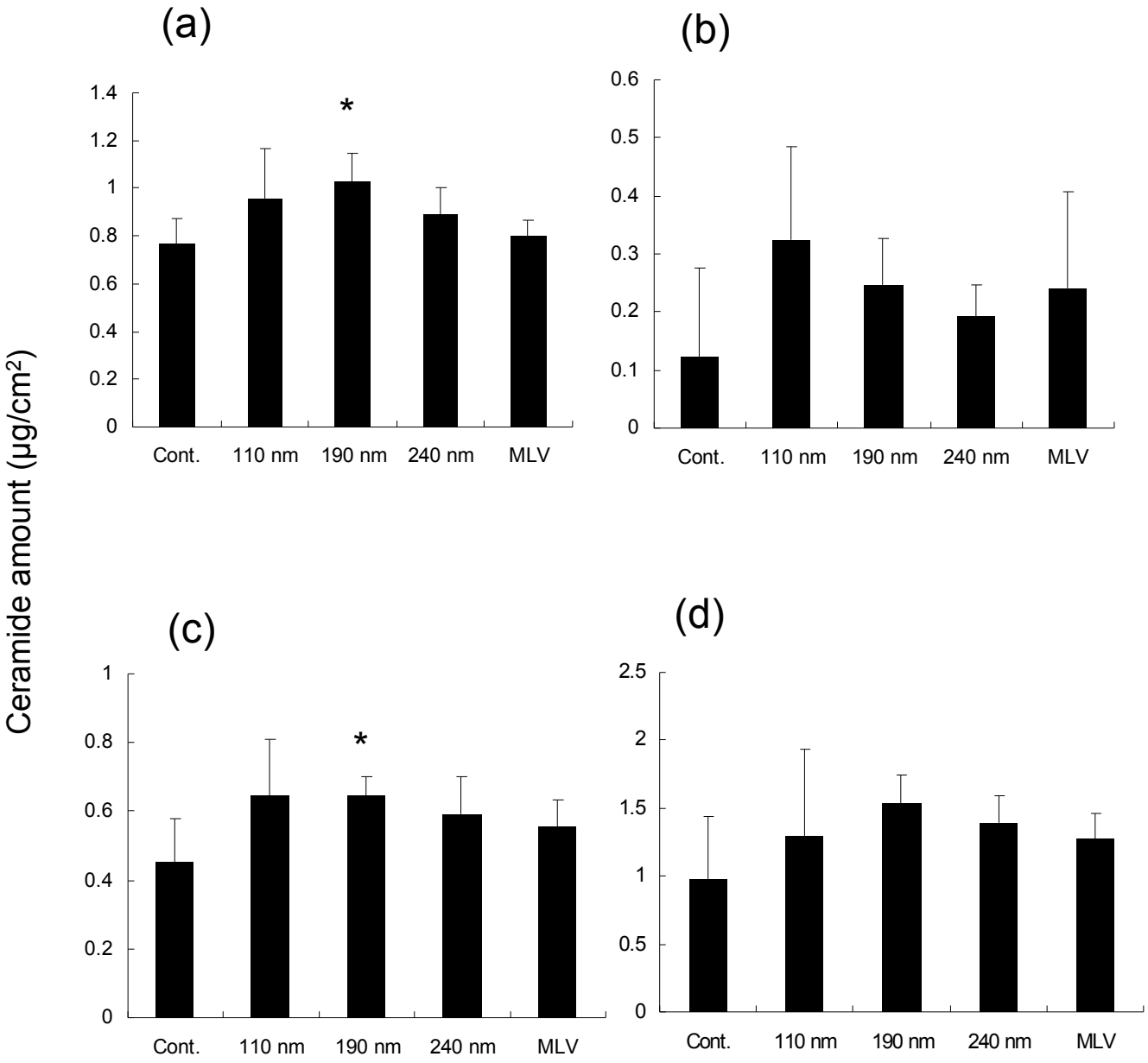


Fig. 2

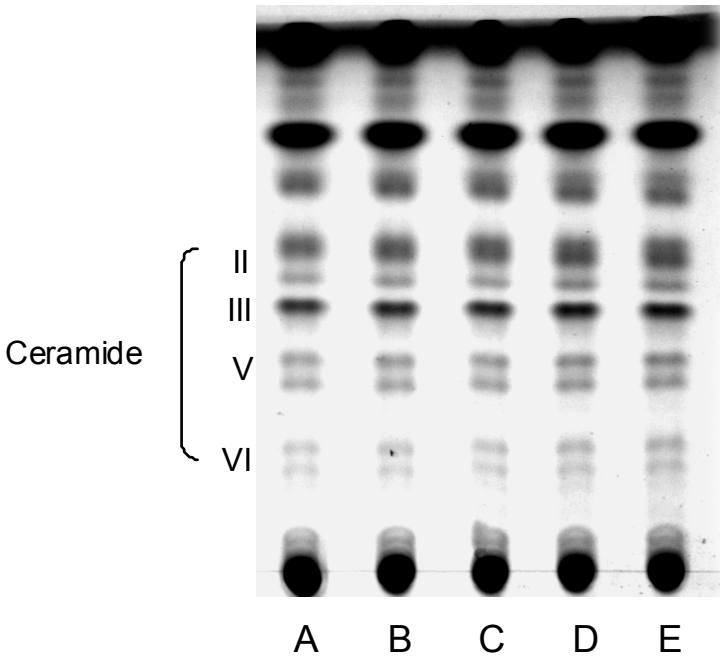


Fig. 3

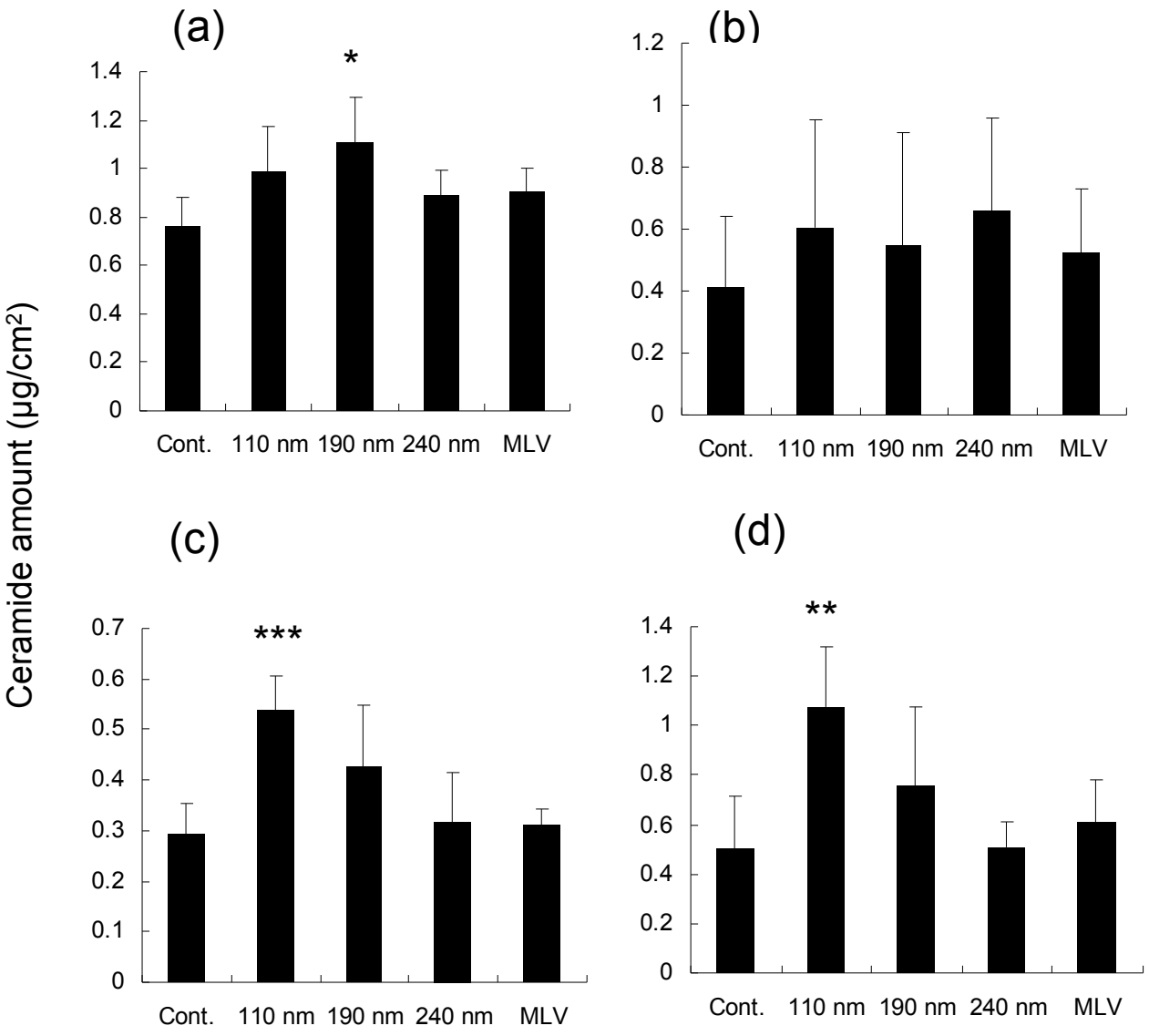

(a)
*
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cont. 110 nm 190 nm 240 nm MLV
(b)
1.2
1
0.8
0.6
0.4
0.2
0
Cont. 110 nm 190 nm 240 nm MLV
Ceramide amount (μg/cm²)
(c)
***
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cont. 110 nm 190 nm 240 nm MLV
(d)
**
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cont. 110 nm 190 nm 240 nm MLV

Fig. 4

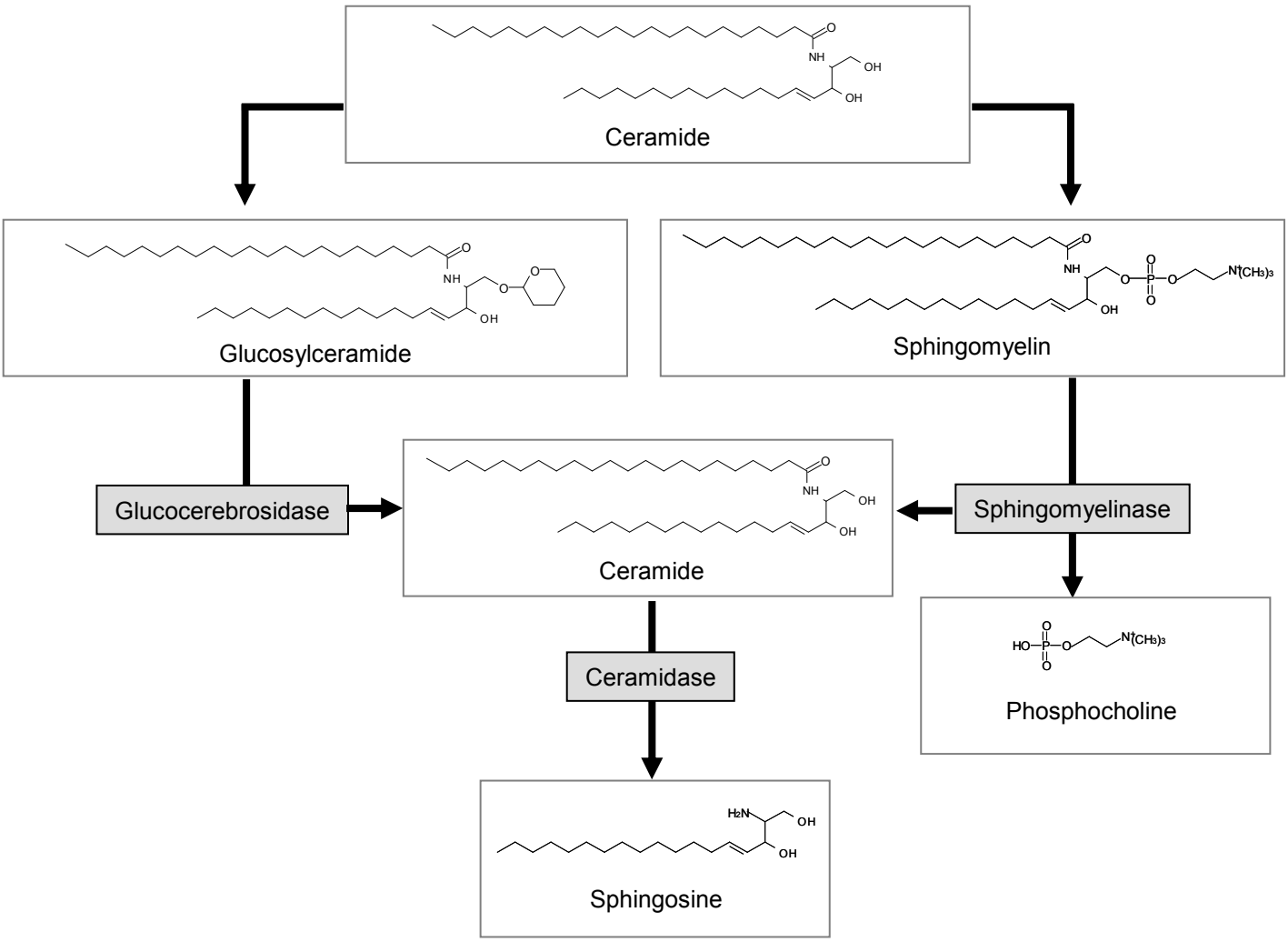

Ceramide
Glucosylceramide
Sphingomyelin
Glucocerebrosidase
Sphingomyelinase
Ceramide
Ceramidase
Phosphocholine
Sphingosine

Fig. 4